

# iPSC-Based Compound Screening and In Vitro Trials Identify a Synergistic Anti-amyloid Combination for Alzheimer ' s Disease

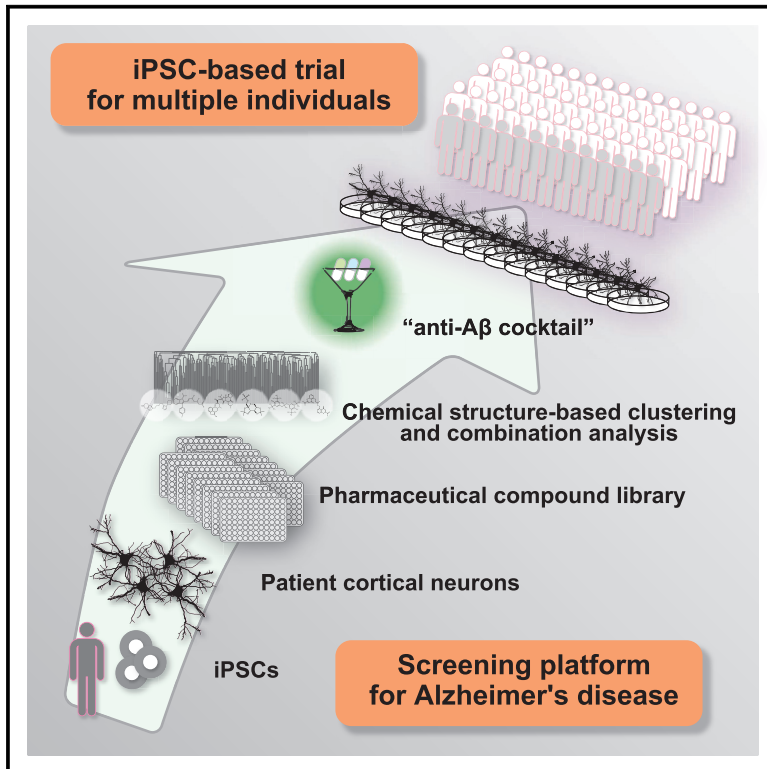
著者	Kondo Takayuki, Imamura Keiko, Funayama Misato, Tsukita Kayoko, Miyake Michiyo, Ohta Akira, Woltjen Knut, Nakagawa Masato, Asada Takashi, Arai Tetsuaki, Kawakatsu Shinobu, Izumi Yuishin, Kaji Ryuji, Iwata Nobuhisa, Inoue Haruhisa
journal or publication title	Cell reports
volume	21
number	8
page range	2304-2312
year	2017-11
権利	(C) 2017 The Author(s). This is an open access article under the CC BY-NC-ND license ( <a href="http://creativecommons.org/licenses/by-nc-nd/4.0/">http://creativecommons.org/licenses/by-nc-nd/4.0/</a> ).
URL	<a href="http://hdl.handle.net/2241/00149338">http://hdl.handle.net/2241/00149338</a>

doi: 10.1016/j.celrep.2017.10.109

# Cell Reports

## iPSC-Based Compound Screening and *In Vitro* Trials Identify a Synergistic Anti-amyloid $\beta$ Combination for Alzheimer's Disease

### Graphical Abstract



### Authors

Takayuki Kondo, Keiko Imamura, Misato Funayama, ..., Ryuji Kaji, Nobuhisa Iwata, Haruhisa Inoue

### Correspondence

haruhisa@cira.kyoto-u.ac.jp

### In Brief

Kondo et al. used human iPSC-derived neurons, which offer human-specific drug responsiveness, for drug development for Alzheimer's disease (AD). Using iPSC-based screening of pharmaceutical compounds and chemical clustering, they found a combination of existing drugs that synergistically improve A $\beta$  phenotypes of AD in cells.

### Highlights

- Rapid, robust neuronal induction from human iPSCs to model AD drug responsiveness
- iPSC-based screening of pharmaceutical compounds for A $\beta$  phenotypes
- A combination of existing drugs synergistically improve A $\beta$  phenotypes of AD
- Anti-A $\beta$  cocktail decreases toxic A $\beta$  levels in neurons derived from patients' cells



# iPSC-Based Compound Screening and *In Vitro* Trials Identify a Synergistic Anti-amyloid $\beta$ Combination for Alzheimer's Disease

Takayuki Kondo,<sup>1,2</sup> Keiko Imamura,<sup>1,2</sup> Misato Funayama,<sup>1</sup> Kayoko Tsukita,<sup>1</sup> Michiyo Miyake,<sup>1,2</sup> Akira Ohta,<sup>1</sup> Knut Woltjen,<sup>1,3</sup> Masato Nakagawa,<sup>1</sup> Takashi Asada,<sup>4</sup> Tetsuaki Arai,<sup>4</sup> Shinobu Kawakatsu,<sup>5</sup> Yuishin Izumi,<sup>6</sup> Ryuji Kaji,<sup>6</sup> Nobuhisa Iwata,<sup>7,8</sup> and Haruhisa Inoue<sup>1,2,9,\*</sup>

<sup>1</sup>Center for iPS Cell Research and Application (CiRA), Kyoto University, Kyoto 606-8507, Japan

<sup>2</sup>Drug-Discovery Cellular Basis Development Team, RIKEN BioResource Center, Kyoto 606-8507, Japan

<sup>3</sup>Hakubi Center for Advanced Research, Kyoto University, Kyoto 606-8501, Japan

<sup>4</sup>Department of Psychiatry, Division of Clinical Medicine, Faculty of Medicine, University of Tsukuba, Ibaraki 305-8575, Japan

<sup>5</sup>Department of Neuropsychiatry, Aizu Medical Center, Fukushima Medical University, Fukushima 969-3492, Japan

<sup>6</sup>Department of Clinical Neuroscience, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima 770-8503, Japan

<sup>7</sup>Department of Genome-based Drug Discovery, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki 852-8521, Japan

<sup>8</sup>Unit for Dementia Research and Drug Discovery, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki 852-8521, Japan

<sup>9</sup>Lead Contact

\*Correspondence: [haruhisa@cira.kyoto-u.ac.jp](mailto:haruhisa@cira.kyoto-u.ac.jp)

<https://doi.org/10.1016/j.celrep.2017.10.109>

## SUMMARY

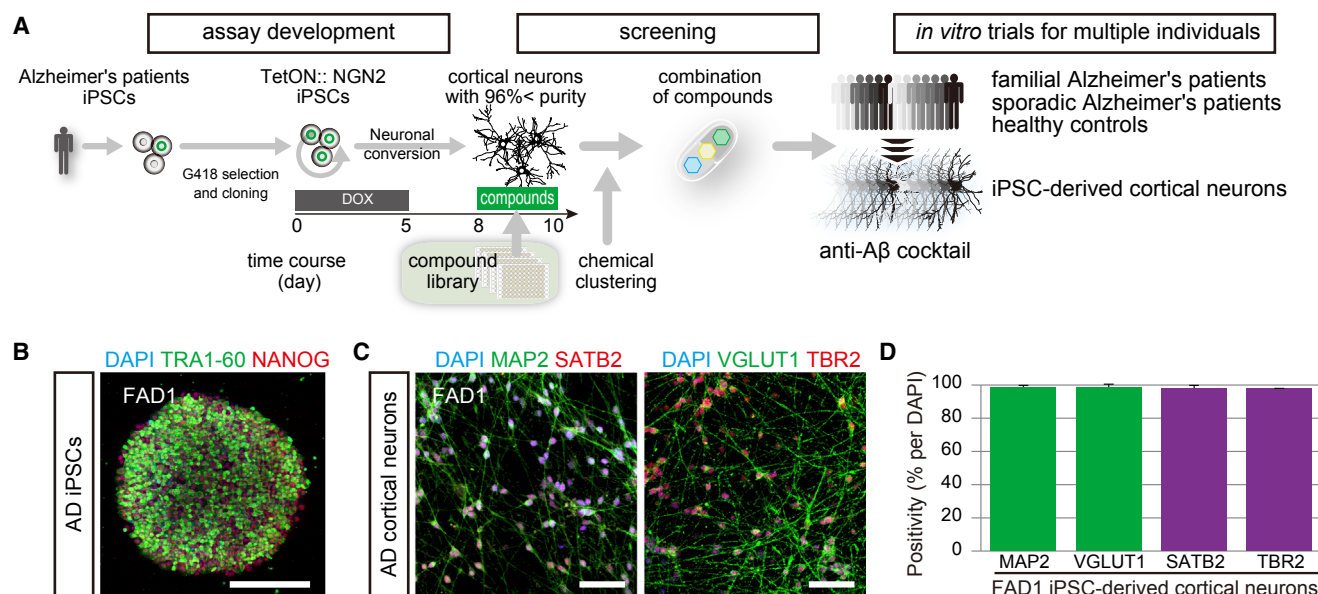
In the process of drug development, *in vitro* studies do not always adequately predict human-specific drug responsiveness in clinical trials. Here, we applied the advantage of human iPSC-derived neurons, which offer human-specific drug responsiveness, to screen and evaluate therapeutic candidates for Alzheimer's disease (AD). Using AD patient neurons with nearly 100% purity from iPSCs, we established a robust and reproducible assay for amyloid  $\beta$  peptide (A $\beta$ ), a pathogenic molecule in AD, and screened a pharmaceutical compound library. We acquired 27 A $\beta$ -lowering screen hits, prioritized hits by chemical structure-based clustering, and selected 6 leading compounds. Next, to maximize the anti-A $\beta$  effect, we selected a synergistic combination of bromocriptine, cromolyn, and topiramate as an anti-A $\beta$  cocktail. Finally, using neurons from familial and sporadic AD patients, we found that the cocktail showed a significant and potent anti-A $\beta$  effect on patient cells. This human iPSC-based platform promises to be useful for AD drug development.

## INTRODUCTION

Human induced pluripotent stem cell (iPSC) technology has revolutionized drug discovery research (Shi et al., 2016) by making it possible to produce diseased cells from patients *in vitro*. In the research field of neurological diseases, direct biopsy of affected tissues from patients causes irreversible injury; therefore, cellular and animal models generated by the transduction and overexpression of disease-causative genes have been

widely used for drug discovery research. However, recent studies have elucidated a large difference in drug responsiveness between human iPSC-derived cells and cancer cell lines (Liu et al., 2014; Mertens et al., 2013; Paull et al., 2015; Yahata et al., 2011). In the process of drug development, the total success rate from hit compounds to final launch is nearly 4.1%, according to some estimates (Paul et al., 2010), and is only 11.6% at clinical trial stages, even after successful preclinical studies. The cause of these low success rates in drug development may, at least partially, be attributed to a difference in drug responsiveness between human beings and other model animals and/or various drug dosages and transgenes that mimic the disease conditions. To minimize this gap, patient-derived iPSCs could be a promising resource for pharmacological research. Here, we modified direct conversion technology (Szabo et al., 2010; Zhang et al., 2013) for neuronal cells from human iPSCs (induced neurons: iNs) and achieved a neuronal cell culture with nearly 100% purity after only a 1-week differentiation period from iPSCs. This extremely pure and rapid method of neuronal differentiation can eliminate the variant efficiency of differentiation among iPSC clones (Onder and Daley, 2012; Thattava et al., 2013) and is more suitable for modeling a pathological condition and compound screening.

We applied this differentiation method to compound screening for Alzheimer's disease (AD), the most common cause of elderly dementia. One of the neuropathological hallmarks of AD is the formation of extracellular amyloid plaques that are composed of aggregated amyloid  $\beta$  peptides (A $\beta$ s) (Powers, 1997; Selkoe, 2004). Extensive studies of human genetics, neuropathology, and model animals indicate that the accumulation of A $\beta$ s is a triggering event that initiates a long-term pathological cascade of AD and eventually leads to dementia (Hardy and Selkoe, 2002; Selkoe, 2002). A $\beta$  is produced by sequential cleavages of amyloid precursor protein (APP) by  $\beta$ -site APP cleaving enzyme 1 (BACE1) and  $\gamma$ -secretase, and these two enzymes have been



**Figure 1. Transient NGN2 Expression Converts iPSCs into Cortical Neurons**

(A) Schema of the differentiation system for iPSC-derived cortical neurons by using the *piggyBac* vector coding human *NGN2* gene, the compound-screening platform, and the *in vitro* trials.

(B) A generated FAD1-iPSC line expressed the pluripotency markers TRA1-60 (green) and NANOG (red). Scale bar, 200  $\mu$ m.

(C) Day 8 FAD1 neurons expressed excitatory cortical neuron markers. Left: MAP2 (green) and SATB2 (red). Right: VGLUT1 (green) and TBR2 (red). Scale bars, 200  $\mu$ m.

(D) Purity of day 8 neurons.

Data indicate mean  $\pm$  SD (n = 3 for each clone).

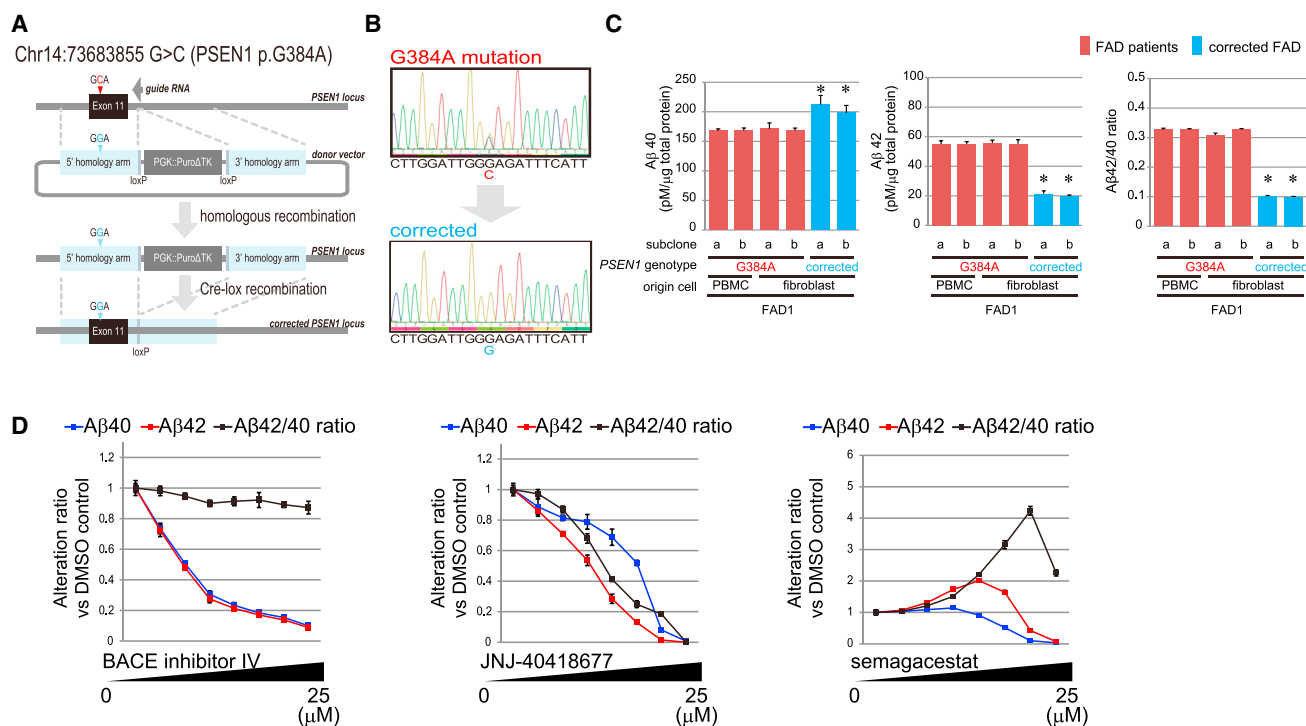
the most important targets for disease-modifying drugs of AD. However, strong inhibition of  $\gamma$ -secretase widely perturbs the processing of numerous endogenous substrates important for physiological functions other than APP, and it has caused serious side effects after long-term treatment (Filsler et al., 2015). After the failure in recent clinical trials of  $\gamma$ -secretase inhibitor, mainly due to on-target side effects in humans (De Strooper, 2014), BACE1 was considered as a more preferable target for anti-A $\beta$  drugs, and several trials of BACE1 inhibitors (BSIs) have been conducted (Barão et al., 2016; Vassar et al., 2014). However, *in vivo* studies, based on BACE1 null mice or BSI treatment have revealed that BACE1 plays roles in several essential neural phenomena, including myelination, ion channel activities, neuronal migration, neuronal excitation, astrogenesis, and muscle spindle activity, among others (Cheret et al., 2013; Hu et al., 2016). Thus, the safety profiles of BSIs should be seriously considered and closely monitored (Barão et al., 2016; Yan, 2016). Furthermore, positron emission tomography (PET) imaging of amyloid and cerebrospinal fluid (CSF) biomarkers, such as the A $\beta$ 42/40 ratio, has shown that amyloid burden in human brains begins about 2 decades before the presentation of overt clinical symptoms of AD (Jack et al., 2013). To prevent AD development, anti-A $\beta$  therapy that is safe and applicable for the long preclinical phase of AD without extensive amyloid burden is desirable. To realize this ideal, we set up a compound screen for a drug-repositioning approach, which has great advantages for research and development costs and time due to enormous post-marketing safety information.

Here, we established a screening platform to explore anti-A $\beta$  compound and combined hit compounds to maximize their anti-A $\beta$  effects. Additionally, we conducted an *in vitro* evaluation study by using iPSC-derived neurons from 13 individuals, including familial and sporadic AD patients, and confirmed that the combination of anti-A $\beta$  compounds could reduce A $\beta$  efficiently in all participants beyond the differences in drug responsiveness among multiple individuals. The models and process of this study should contribute to overcoming possible drawbacks of drug discovery and development by a standard platform using human iPSCs.

## RESULTS

### Robustly Differentiated Cortical Neurons Ensure Fine A $\beta$ Phenotypes

Technically, the lack of consistent differentiation efficacy with a high purity of iPSCs to neurons has been an issue for precise modeling of the pathological condition and subsequent drug screening (Inoue et al., 2014). To overcome this issue, we utilized direct conversion technology to differentiate human iPSCs into cortical neurons (Davis et al., 1987; Vierbuchen et al., 2010) (Figure 1A). Direct neural conversion using lentiviral induction of neurogenin 2 (*NGN2*) was reported to provide mature neurons with 75%–100% purity (MAP2 [microtubule-associated protein 2]-positive cells for lentivirally infected GFP-positive cells) (Zhang et al., 2013), but the total neuronal purity (neurons per total cells in a dish) depends on the efficiency of the lentiviral infection. To



**Figure 2. Alzheimer's Disease Patient Cortical Neurons Showed A $\beta$  Phenotypes, Corrected by Genome Editing**

(A) Schema of genome editing for *PSEN1* exon11, using the CRISPR-Cas9 system.

(B) Sanger-sequence data of the genome-corrected site in *PSEN1* G384A.

(C) A $\beta$  phenotypes of iPSC-derived cortical neurons. Data indicate mean  $\pm$  SD ( $n = 3$  for each clone; \* $p < 0.05$ , Dunnett's test for multiple comparisons to FAD1 PBMC origin).

(D) ELISA quantification of A $\beta$  species, altered by adding BSI-IV ( $\beta$ -secretase inhibitor), JNJ-40418677 ( $\gamma$ -secretase modulator), or semagacestat ( $\gamma$ -secretase inhibitor). Plots show the results of serial 5-fold dilutions ranging from 1.6 nM to 25  $\mu$ M of the respective compounds. Data indicate mean  $\pm$  SD ( $n = 3$  for each concentration).

transduce NGN2 into all cells in a dish, we established human iPSC clones with doxycycline-inducible human NGN2 from a familial AD patient bearing a heterozygous G384A mutation of the *PSEN1* gene, which encodes presenilin-1, by using *piggyBac* transposon (Figure 1B) (Kim et al., 2016). We tested 5-day NGN2 expression via genome-integrated *piggyBac* vector and found that the cortical neurons induced from iPSCs had more than 96% purity (Figures 1C and 1D) and expressed no remaining exogenous NGN2 (Figure S1A) on day 8. Further, the induced cortical neurons were electrophysiologically functional (Figure S1B).

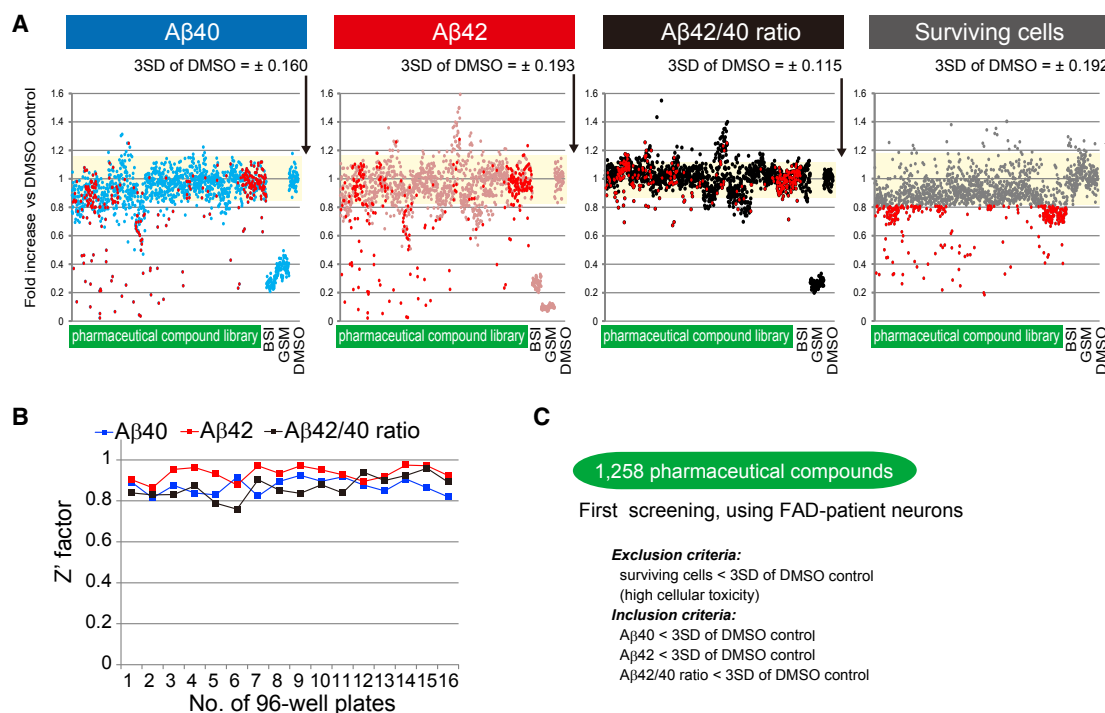
Mutations of *PSEN1* are known to increase the production of A $\beta$ 42 as a toxic A $\beta$  species, and the A $\beta$ 42/40 ratio is an index of A $\beta$  toxicity (Citron, 2010), compared with wild genotype (Page et al., 2008). To confirm that the system developed in this study can recapitulate the A $\beta$  phenotypes of *PSEN1* mutation precisely, we corrected the *PSEN1* G384A mutation of FAD1 by using CRISPR-Cas9 technology (Figures 2A and 2B). Cortical neurons with a heterozygous G384A mutation in *PSEN1* produced a larger amount of A $\beta$ 42 and showed a higher A $\beta$ 42/40 ratio compared to that of neurons after genome correction (Figure 2C). Recapitulated A $\beta$  phenotypes of FAD1 were similar to those of different iPSC clones that originated from a different type of FAD1 somatic cells (Figures 2C, S2A, and

S2B). These results confirmed that our A $\beta$  assay can provide precise phenotypes of FAD and that it offers reproducible evaluation of different iPSC clones. We also validated our assay system by applying commercially available A $\beta$  production-modifying compounds, including  $\beta$ -secretase inhibitor IV (BSI-IV), JNJ-40418677 (second-generation  $\gamma$ -secretase modulator: GSM), and semagacestat ( $\gamma$ -secretase inhibitor: GSI) as positive controls and confirmed the inhibitory effects of the compounds on A $\beta$  production (Figure 2D). On the other hand, paradoxically, a low concentration of semagacestat increased A $\beta$ 42, and a low concentration of non-steroidal anti-inflammatory drugs (NSAIDs), which are first-generation GSMs, failed to improve A $\beta$  levels (Figure S2C), which is consistent with previous reports (Liu et al., 2014; Mertens et al., 2013; Yahata et al., 2011). From these results, we confirmed the establishment of a robust and reproducible screening system to assess changes in A $\beta$  production in response to test compounds.

### Screening for Anti-A $\beta$ Compounds Using a Pharmaceutical Compound Library

We screened a compound library that consists of 2  $\mu$ M each of 1,258 pharmaceutical compounds and ran tests to find anti-A $\beta$  compounds as a first-step screening (Figure 3A). We defined 0.1% DMSO as a baseline control, 2  $\mu$ M BSI-IV as a positive





**Figure 3. First-Step Screening of a Pharmaceutical Compound Library**

(A) Scatterplot graphs of the first-step screening. Fold changes compared with DMSO control were plotted according to each analyte, including Aβ40 (blue), Aβ42 (red), the Aβ42/40 ratio (black), and surviving cells (gray), by adding hit compounds or positive control compounds. The 3SD variance of DMSO control groups is indicated by the yellow band. Compounds causing less survival than 3SD of DMSO control are indicated by overlapping deep red dots. BSI, β-secretase inhibitor; GSM, γ-secretase modulator.

(B) Points represent the Z' factor of each 96-well plate in the first-step screening.

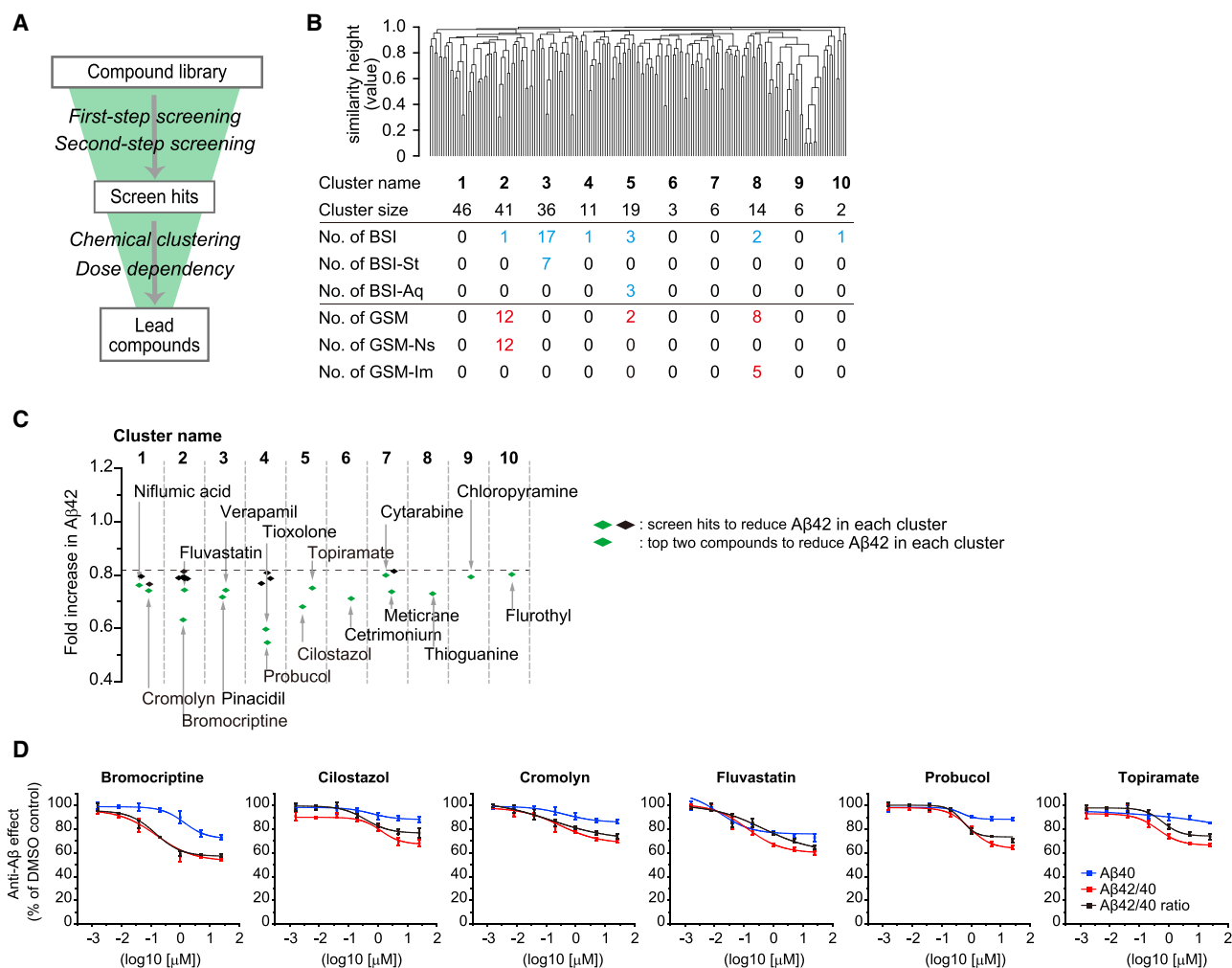
(C) Criteria of the first-step screening.

control for alteration in Aβ40 production, and 2 μM JNJ-40418677 as a positive control for alteration in Aβ42 production and the Aβ42/40 ratio alteration in each assay using a 96-well-plate. Through all screening sets, Z' factor, an indicator of screening feasibility and reproducibility, was suitably high—more than 0.8—in each analyte (Figure 3B). The coefficient of variation (%CV) of DMSO control in each assay plate was below 5% (Figure S3A). From these results, we could confirm that this screening system successfully assessed dynamic Aβ responses and had low variability among the assay sets. We set the inclusion criteria of the first-step screening at “more than threefold of standard deviation values (3SD) of DMSO control in each analyte” to collect a wide range of potential anti-Aβ compounds (below the yellow field in the Figure 3A graph of Aβ40, Aβ42, and the Aβ42/40 ratio). To leave out toxic compounds that could ostensibly lead to Aβ reduction from a decreased number of neurons, we excluded compounds that caused cell survival to be less than 3SD of DMSO control (red dots in Figure 3A). According to the inclusion and exclusion criteria of the first-step screening, we collected 129 compounds (Figure 3C; Data S1). In the next step, to set stringent hit criteria, we tested the 129 compounds on both FAD1 iPSC-derived neurons and another iPSC-derived neurons, originating from peripheral blood mononuclear cells (PBMCs) of FAD1 (named “PBMC origin”). To confirm the

screening reproducibility, we measured again the anti-Aβ effects of 129 compounds at 1 μM each as the second-step screening by plotting the fold-change in Aβ42, a toxic Aβ with a higher propensity to form insoluble Aβ in amyloid plaques of AD brain (Walsh et al., 2002). We observed a high correlation between FAD1 fibroblast-origin and FAD1 PBMC-origin neurons (Figure S3B). As a result, we could confirm the reproducibility of the developed screening system between different iPSC clones, and finally selected 27 screen hits that passed the inclusion criteria of Aβ42.

### Chemical Structure Clustering Identified Six Lead Compounds

The anti-Aβ effects of the 27 screen hits were not as strong as those of known BSIs, GSIs, or GSIs. To select synergistic combinations with maximal anti-Aβ effects, we attempted to classify and prioritize hit compounds based on fingerprinting of the compound chemical structure (Figures 4A and S4A). The fingerprinting technique is widely used, and it successfully detects structurally diverse active compounds of various similarity levels (Gardiner et al., 2011; Vogt et al., 2010). We converted the chemical structures of 129 compounds after the first-step screening along with those of known BSIs, known GSIs, and known GSIs (Data S2) into the fingerprinting format by using the



**Figure 4. Chemical Structure Clustering Identified Six Lead Compounds**

(A) Schema of the screening steps.

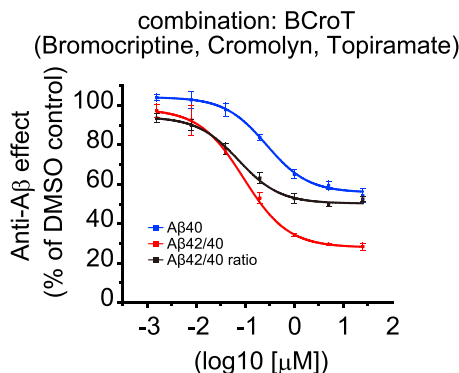
(B) Chemical-structure clustering classified 129 compounds, filtered by first-step screening, and 54 known A $\beta$  modifiers into 10 clusters, based on ECFP4 fingerprint similarity. The small table shows the number of compounds in each cluster, or known-A $\beta$  modifiers in each cluster, including BSIs or GSMs. BSI-St, statin-derived BSI; BSI-Aq, aminoquinazoline-based BSI; GSM-Ns, NSAIDs-based GSMs; GSM-Im, GSM with imidazole structure.

(C) The A $\beta$ 42 alteration ratio (versus DMSO control) of 27 screen hits is shown in the scatterplot graph according to each cluster. Each compound name of the top two compounds to reduce A $\beta$ 42 in each cluster is labeled.

(D) The selected six anti-A $\beta$  compounds decreased A $\beta$  production in a dose-dependent manner. Data indicate mean  $\pm$  SD ( $n = 3$  for each clone).

Extended Connectivity Fingerprints method ([ECFP] version 4; ECFP4), which is suitable for computer processing (Rogers and Hahn, 2010). We divided various fingerprints into ten clusters by calculating a measure of molecular similarity using the Tanimoto coefficient (Tc), which is the gold standard in the chemoinformatics field (Willett et al., 1998). Tc is a numerical measure of similarity ranging from zero (no fingerprint overlap) to one (fingerprint identity). A high Tc among different compounds has generally shown similar pharmacological activities in several studies (Jasial et al., 2016). Fingerprints of the compounds were examined for any similarity of structural formulas through clustering analysis based on a distance matrix (Figure 4B). After clustering, we could separate the BSIs with similar structures into one group; for instance, statin-derived BSIs (BSI-Sts) into cluster

3 or aminoquinazoline-based BSIs (BSI-Aqs) into cluster 5. We could also separate the GSMs with similar structures into one group; for instance, NSAID-based GSMs (GSM-Ns) into cluster 2 or GSMs with imidazole structure (GSM-Im) into cluster 8. For these investigations, we successfully carried out non-biased chemical structure clustering and classified the compounds and known A $\beta$ -processing modifiers into ten groups (Figure 4C). To select potent anti-A $\beta$  compounds from each group, we selected the top two compounds from each cluster and investigated whether dose-dependent reactivity to these compounds could be seen (Figure 4C). We found 11 compounds with dose-dependency A $\beta$ 42 reduction and excluded 5 compounds because they caused massive cell death at higher concentrations of 5–25  $\mu$ M (Figure S4B). Finally, six compounds (bromocriptine, cilostazol,



**Figure 5. Combination of Anti-Aβ Compounds Improved Alzheimer's Disease Phenotypes**

Combination of anti-Aβ compounds (BCroT) decreased Aβ production in a dose-dependent manner. Data indicate mean  $\pm$  SD ( $n = 3$  for each clone). BCroT, combination of bromocriptine, cromolyn, and topiramate.

cromolyn, fluvastatin, probucol, and topiramate) that showed dose-dependent Aβ42 reduction and high maximum effect ( $E_{max}$ ) (more than  $-0.25$ ) were chosen as lead compounds (Figure 4D). All but bromocriptine have been reported to show *in vivo* anti-Aβ effects (Table S1). However, we identified bromocriptine as having the most potent anti-Aβ effect. We conducted additional experiments on bromocriptine to investigate the candidate structure that modifies Aβ metabolism. First, we evaluated various dopamine receptor stimulants and determined that only bromocriptine, a specific agonist to dopamine receptor D2 subtype (DRD2), could alter Aβ metabolism (Figure S4C). Next, we compared the effect on Aβ metabolism between DRD2 agonists that include and those that exclude the ergoline structure. Potent DRD2 agonists without the ergoline structure, including talipexole and pramipexole, did not alter Aβ metabolism (Figure S4D). On the other hand, DRD2 agonists with the ergoline structure, including bromocriptine, pergolide, and cabergoline, altered Aβ metabolism (Figure S4D). These results revealed that the ergoline structure, and not the DRD2-agonistic effect, is important for altering the Aβ metabolism of human neurons. Additionally, we confirmed that compounds with the ergoline structure, which have a more than 80% structure similarity to bromocriptine, alter Aβ metabolism in a dose-dependent manner (Figure S4E), and we found that the ergot-alkaloid structure, which contains a tripeptide structure, acts to reduce Aβ production. From these results, our screening system of patient iPSC-derived neurons could select appropriate hits on the basis of the anti-Aβ effect from a compound library.

### A Combination of Three Compounds Can Decrease Toxic Aβ

To maximize the anti-Aβ effects, we combined the six lead compounds, all of which have different structures. We analyzed every possible combination of the six compounds (Figure S5) and identified a combination of three (bromocriptine, cromolyn, topiramate [BCroT]) as the most potent anti-Aβ combinations. These three compounds belonged to different clusters (Figure 4C). Examining the dose-dependent curves of BCroT for Aβ42, we

found a half maximal effective concentration ( $EC_{50}$ ) value of  $1.0 \mu M$ , and  $E_{max}$  values exceeding 70% reduction were seen (Figure 5).

### Surveying Pharmacological Responses of Multiple Individuals

Up to this point, the analysis was conducted using FAD neurons with the *PSEN1* G384A mutation (clone name FAD1). To demonstrate the effect of BCroT on expanded populations, we conducted *in vitro* studies by using iPSCs from multiple individuals. We carried out the evaluation by using four additional iPSCs with AD-causative mutations in *PSEN1* or *APP* (clone name "FAD"), four sporadic AD iPSCs (clone name "SAD"), and six control iPSCs (clone name "HC" or "Corrected") (Figure 6A). The established human iPSCs showed pluripotency markers (Figure S6A) and could be converted into cortical neurons with more than 96% purity (Figures S6B–S6D). The combination of anti-Aβ compounds in the cocktail successfully showed more than 30% reduction in both Aβ40 and Aβ42 levels in all clones (Figures 6B) and also a reduced high Aβ42/40 ratio of *PSEN1*-mutated FADs (Figure 6B). From these results, we could highlight the combination of existing drugs as a new potential anti-Aβ cocktail for AD.

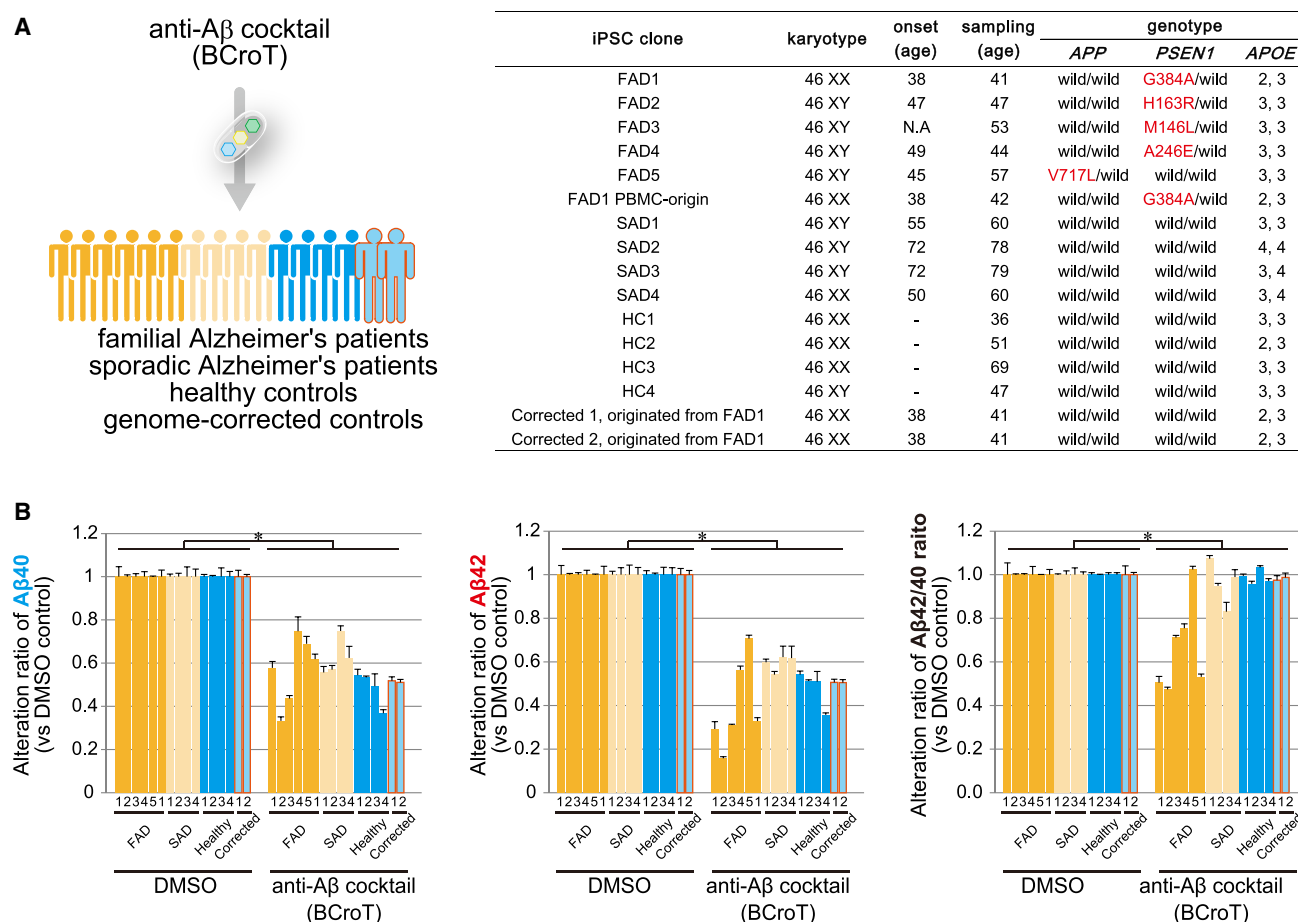
### DISCUSSION

In this study, we have developed a robust and rapid method of neural induction from human iPSCs. Using these cells, we screened pharmaceutical compounds and found that BCroT attenuated Aβ phenotypes efficiently. Finally, we confirmed the efficacy of the anti-Aβ cocktail using iPSC-derived neurons from multiple AD patients. Accordingly, we propose a platform of drug discovery and development using human iPSCs and compound screening.

Since AD prevention requires early intervention and long-term usable drugs with validated safety, we screened pharmaceutical compounds to identify anti-Aβ compounds, although hit compounds were predicted to show a relatively weak effect on Aβ metabolism, compared with direct inhibitors of  $\beta$ - or  $\gamma$ -secretase. Therefore, we combined screen hits having different action sites to gain synergistic effects. For this purpose, we conducted chemical clustering of the hit structures, based on the hypothesis that compounds with different chemical structures target different molecules. We selected 6 compounds and identified BCroT as having synergistic anti-Aβ effects. These results suggested that the chemical clustering approach is useful for determining synergistic combinations of compounds. Additionally, we found that the stimulation of dopamine receptors, which are targets of bromocriptine, did not alter Aβ metabolism, and we identified the ergoline ring as a key structure for reducing Aβ production. These results indicated that bromocriptine may reduce the Aβ level by modifying the targets of ergot alkaloids (Wallwey and Li, 2011).

The anti-Aβ cocktail of BCroT possessed a potent inhibitory effect on Aβ production in cortical neurons from AD patients with mutant *PSEN1* and effectively diminished toxic Aβ to less than 40%, which is the same level achieved with general BSI or GSM treatment. At the same time, BCroT showed a modest





**Figure 6. Anti-A $\beta$  Cocktail of BCroT Decreased Toxic A $\beta$  in Cortical Neurons from a Variety of Individuals**

(A) Left: schema of the *in vitro* trials of BCroT in human iPSC-derived neurons from multiple individuals. BCroT, combination of bromocriptine, cromolyn, and topiramate. Right: patient information and generated iPSC characters. PBMC, peripheral blood mononuclear cell; FAD, familial Alzheimer's disease; SAD, sporadic Alzheimer's disease; HC, healthy control.

(B) BCroT suppressed the production of A $\beta$ 40 (left), A $\beta$ 42 (middle), and the A $\beta$ 42/40 ratio (right), even in FAD and SAD neurons other than FAD1 neurons. Data indicate mean  $\pm$  SD ( $n = 3$  for each clone) ( $n = 16$  clones for each group;  $^*p < 0.05$ ).

effect on cortical neurons from sporadic AD and healthy control iPSCs, with a 20%–30% reduction of toxic A $\beta$  levels. A recent Icelandic genome-cohort study revealed that the APP A673T mutation located adjacent to the  $\beta$ -cleavage site showed a 20%–30% reduction in A $\beta$  production and was resistant to the onset of AD (Jonsson et al., 2012), suggesting that the modest effect of the anti-A $\beta$  cocktail, BCroT, could be sufficient to prevent AD development if given to preclinical-AD patients. Furthermore, the difference of drug responsiveness between familial AD with PSEN1 mutation and sporadic AD indicates that the individual genetic background contributes to the responsiveness, and thus, in the future, personalized compound screening would be useful for identifying more effective compounds.

In this study, we used pure cortical neurons differentiated from human iPSCs for the compound screening. This approach ignores interactions of these neurons with vascular cells, glial cells, and the blood-brain barrier. For this reason, we were unable to translate the anti-A $\beta$  effects directly to the clinical study. With this in mind, mixed cultures of multiple cell types would be valu-

able for mimicking the complexity of the brain. Furthermore, when applying *in vitro* results to *in vivo* efficacy, the brain bioavailability of orally administered compounds should be considered. Bromocriptine and topiramate are known to be efficiently delivered into brain, and cromolyn can alter brain phenotypes in various animal models (Hori et al., 2015; San-Martín-Clark et al., 1995). However, pharmacokinetic data of the anti-A $\beta$  cocktail BCroT are not available, and it is difficult to recapitulate the absorption, degradation, and clearance of compounds through the whole body with an *in vitro* model. In future studies, the administration of the anti-A $\beta$  cocktail BCroT to mice with AD might provide direct *in vivo* evidence for feasible clinical trials.

## EXPERIMENTAL PROCEDURES

### Ethical Approval

This study was approved by the Ethics Committee of the Graduate School and Faculty of Medicine, Kyoto University, and the Kyoto University Hospital (approval numbers R0091 and G259).

### Generation of iN-iPSCs

To establish a robust and rapid differentiation method, we utilized direct conversion technology. Human NGN2 cDNA, under tetracycline-inducible promoter (tetO), was transfected into iPSCs by a *piggyBac* transposon system (Kim et al., 2016) and Lipofectamine LTX (Thermo Fisher Scientific, Waltham, MA). We mainly used the vector containing tetO::NGN2 (Figure 1A) and, additionally, used another vector containing TetO::NGN2-IRES-mCherry only to evaluate the time-dependent decrease of the transgenes (Figure S1B). After antibiotic selection of G418 disulfate (Nacalai-Tesque, Kyoto, Japan), we picked out colonies and selected subclones that could efficiently differentiate into neurons by inducing the temporal expression of NGN2, with MAP2/DAPI purity > 96%.

### First-Step Screening

On day 0, iN-iPSCs were dissociated with TrypLE Express (GIBCO, Thermo Fisher Scientific) and disseminated on a mixed coating of poly-L-lysine (final 0.0002% v/w, Sigma Aldrich, Japan), Corning Synthemax II-SC (final 20 µg/mL, Corning, NY), and Matrigel (final 2% v/v, Corning). Disseminated iPSCs were cultured in Neurobasal Medium (GIBCO, Thermo Fisher Scientific) supplemented with 0.5% B27 without vitamin A (GIBCO, Thermo Fisher Scientific), 1× Glutamax (GIBCO, Thermo Fisher Scientific), 2 µg/mL doxycycline hydrochloride (Wako Pure Chemicals Industries, Japan), and 5 µM Y-27632 (Nacalai-Tesque) from day 0 to day 5. On day 5, differentiated neural cells were disseminated into 96-well plates by passive humidity control (Nunc Edge plates, Thermo Fisher Scientific), which can eliminate the evaporation of culture medium and minimize well-to-well variability. Disseminated neural cells were cultured in Neurobasal Medium supplemented with 0.5% B27 without vitamin A and 1× Glutamax from day 5 to day 8. On day 8, all culture media were replaced with 120 µL fresh medium, containing each of the 2 µM of each compound in final 0.1% DMSO carrier or only 0.1% DMSO. Neurons or culture media were subjected to analysis 48 hr later.

### Pharmaceutical Compound Library

We used the Microsource International Drug and Microsource US Drug library, which includes a total of 1,258 pharmaceutical compounds that have reached clinical trial stages in the United States. Each compound has been assigned United States Adopted Names (USAN) or United States Pharmacopeial (USP) status and is included in the USP Dictionary. Each 96-well plate contained 80 compounds per plate, four positive controls for Aβ40 (2 µM BSI-IV), four positive controls for Aβ42 and the Aβ42/40 ratio (2 µM JNJ-40418677), and eight negative controls (0.1% DMSO carrier). The raw data of each compound or positive control were normalized to calculate the alteration ratio by using the average data of the eight DMSO controls in each plate (the alteration ratio = raw data of each compound/averaged data of eight DMSO controls in each plate).

### Structure-Based Clustering of Chemical Compounds

We prepared a list of additional chemical compounds, including (1) the first-step screening active compounds (n = 129) and (2) previously reported β- or γ-secretase modifier compounds (n = 55). We classified the total 184 compounds into 10 hierarchical clusters based on similarity (entrusted to Kyoto Constella Technologies, Japan). The similarity (Tc: Tanimoto coefficient) among compounds is defined using the ECFP4 fingerprint method (Rogers and Hahn, 2010), as calculated by jCompoundMapper (Hinselmann et al., 2011). The distance was defined as “Distance = 1 – Tc.” We decided on a comprehensive distance matrix among all 184 compounds and finally classified compounds into 10 similar hierarchical clusters by the furthest neighbor method, using the statistical software tool “R.”

### Statistical Analysis

All data are indicated as mean ± SD. For comparisons of the mean between two groups, statistical analysis was performed using a two-tailed Student's t test. For comparisons of the mean among more than three groups, statistical analysis was performed using a one-way ANOVA, followed by a post hoc test using Dunnett's test. All analyses were performed by using JMP 9 software (SAS Institute, Cary, NC). p values < 0.05 were considered significant.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, one table, and two data files and can be found with this article online at <https://doi.org/10.1016/j.celrep.2017.10.109>.

### AUTHOR CONTRIBUTIONS

H.I. conceived the project. T.K., N.I., and H.I. designed the experiment. T.K., K.I., M.F., K.T., M.M., and H.I. performed the experiments and analyzed the data. K.I. performed neurophysiological procedures. A.O. provided compound libraries. K.W. and M.N. provided the materials and supervised the vector constructions and iPSC cultivation method. S.K., T. Asada, T. Arai, Y.I., and R.K. provided patient samples and information. T.K., N.I., and H.I. wrote the manuscript.

### ACKNOWLEDGMENTS

We would like to express our sincere gratitude to all our coworkers and collaborators; to Takako Enami and Ran Shibukawa for technical assistance; to Peter Karagiannis for critical reading and editing of the manuscript; and to Noriko Endo and Ruri Taniguchi for their valuable administrative support. This research was funded in part by the Research Project for Practical Applications of Regenerative Medicine from AMED (to H.I.); a grant from the Core Center for iPSC Research of Research Center Network for Realization of Regenerative Medicine from AMED (to H.I.); and a JSPS KAKENHI Grant-in-Aid for Young Scientists (B) 17K16121 (to T.K.). The experimental protocols dealing with human or animal subjects were approved by the institutional review board of each institute.

Received: February 24, 2016

Revised: September 17, 2017

Accepted: October 26, 2017

Published: November 21, 2017

### REFERENCES

- Barão, S., Moechars, D., Lichtenthaler, S.F., and De Strooper, B. (2016). BACE1 physiological functions may limit its use as therapeutic target for Alzheimer's disease. *Trends Neurosci.* 39, 158–169.
- Cheret, C., Willem, M., Fricker, F.R., Wende, H., Wulf-Goldenberg, A., Tahir-ovic, S., Nave, K.-A., Saftig, P., Haass, C., Garratt, A.N., et al. (2013). Bace1 and Neuregulin-1 cooperate to control formation and maintenance of muscle spindles. *EMBO J.* 32, 2015–2028.
- Citron, M. (2010). Alzheimer's disease: strategies for disease modification. *Nat. Rev. Drug Discov.* 9, 387–398.
- Davis, R.L., Weintraub, H., and Lassar, A.B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* 51, 987–1000.
- De Strooper, B. (2014). Lessons from a failed γ-secretase Alzheimer trial. *Cell* 159, 721–726.
- Filser, S., Ovsepian, S.V., Masana, M., Blazquez-Llorca, L., Brandt Elvang, A., Volbracht, C., Müller, M.B., Jung, C.K.E., and Herms, J. (2015). Pharmacological inhibition of BACE1 impairs synaptic plasticity and cognitive functions. *Biol. Psychiatry* 77, 729–739.
- Gardiner, E.J., Holliday, J.D., O'Dowd, C., and Willett, P. (2011). Effectiveness of 2D fingerprints for scaffold hopping. *Future Med. Chem.* 3, 405–414.
- Hardy, J., and Selkoe, D.J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297, 353–356.
- Hinselmann, G., Rosenbaum, L., Jahn, A., Fechner, N., and Zell, A. (2011). jCompoundMapper: An open source Java library and command-line tool for chemical fingerprints. *J. Cheminform.* 3, 3.
- Hori, Y., Takeda, S., Cho, H., Wegmann, S., Shoup, T.M., Takahashi, K., Irimia, D., Elmaleh, D.R., Hyman, B.T., and Hudry, E. (2015). A Food and Drug Administration-approved asthma therapeutic agent impacts amyloid β in the brain in a transgenic model of Alzheimer disease. *J. Biol. Chem.* 290, 1966–1978.

- Hu, X., Fan, Q., Hou, H., and Yan, R. (2016). Neurological dysfunctions associated with altered BACE1-dependent Neuregulin-1 signaling. *J. Neurochem.* 136, 234–249.
- Inoue, H., Nagata, N., Kurokawa, H., and Yamanaka, S. (2014). iPS cells: a game changer for future medicine. *EMBO J.* 33, 409–417.
- Jack, C.R., Jr., Knopman, D.S., Jagust, W.J., Petersen, R.C., Weiner, M.W., Aisen, P.S., Shaw, L.M., Vemuri, P., Wiste, H.J., Weigand, S.D., et al. (2013). Tracking pathophysiological processes in Alzheimer's disease: an updated hypothetical model of dynamic biomarkers. *Lancet Neurol.* 12, 207–216.
- Jasial, S., Hu, Y., Vogt, M., and Bajorath, J. (2016). Activity-relevant similarity values for fingerprints and implications for similarity searching. *F1000Res* 5, 591.
- Jonsson, T., Atwal, J.K., Steinberg, S., Snaedal, J., Jonsson, P.V., Björnsson, S., Stefansson, H., Sulem, P., Gudbjartsson, D., Maloney, J., et al. (2012). A mutation in APP protects against Alzheimer's disease and age-related cognitive decline. *Nature* 488, 96–99.
- Kim, S.I., Ocegüera-Yanez, F., Sakurai, C., Nakagawa, M., Yamanaka, S., and Woltjen, K. (2016). Inducible transgene expression in human iPS cells using versatile all-in-one piggyBac transposons. *Methods Mol. Biol.* 1357, 111–131.
- Liu, Q., Waltz, S., Woodruff, G., Ouyang, J., Israel, M.A., Herrera, C., Sarsoza, F., Tanzi, R.E., Koo, E.H., Ringman, J.M., et al. (2014). Effect of potent  $\gamma$ -secretase modulator in human neurons derived from multiple presenilin 1-induced pluripotent stem cell mutant carriers. *JAMA Neurol.* 71, 1481–1489.
- Mertens, J., Stüber, K., Wunderlich, P., Ladewig, J., Kesavan, J.C.C., Vandenberghe, R., Vandenbulcke, M., van Damme, P., Walter, J., Brüstle, O., and Koch, P. (2013). APP processing in human pluripotent stem cell-derived neurons is resistant to NSAID-based  $\gamma$ -secretase modulation. *Stem Cell Reports* 1, 491–498.
- Onder, T.T., and Daley, G.Q. (2012). New lessons learned from disease modeling with induced pluripotent stem cells. *Curr. Opin. Genet. Dev.* 22, 500–508.
- Page, R.M., Baumann, K., Tomioka, M., Pérez-Revuelta, B.I., Fukumori, A., Jacobsen, H., Flohr, A., Luebbbers, T., Ozmen, L., Steiner, H., and Haass, C. (2008). Generation of A $\beta$ <sub>38</sub> and A $\beta$ <sub>42</sub> is independently and differentially affected by familial Alzheimer disease-associated presenilin mutations and  $\gamma$ -secretase modulation. *J. Biol. Chem.* 283, 677–683.
- Paul, S.M., Mytelka, D.S., Dunwiddie, C.T., Persinger, C.C., Munos, B.H., Lindborg, S.R., and Schacht, A.L. (2010). How to improve R&D productivity: the pharmaceutical industry's grand challenge. *Nat. Rev. Drug Discov.* 9, 203–214.
- Paull, D., Sevilla, A., Zhou, H., Hahn, A.K., Kim, H., Napolitano, C., Tsankov, A., Shang, L., Krumholz, K., Jagadeesan, P., et al. (2015). Automated, high-throughput derivation, characterization and differentiation of induced pluripotent stem cells. *Nat. Methods* 12, 885–892.
- Powers, J.M. (1997). Diagnostic criteria for the neuropathologic assessment of Alzheimer's disease. *Neurobiol. Aging* 18 (4, Suppl), S53–S54.
- Rogers, D., and Hahn, M. (2010). Extended-connectivity fingerprints. *J. Chem. Inf. Model.* 50, 742–754.
- San-Martín-Clark, O., Cuéllar, B., De Alba, J., Leza, J.C., and Lorenzo, P. (1995). Changes induced by sodium cromoglycate in brain catecholamine turnover in morphine dependent and abstinent mice. *Psychopharmacology (Berl.)* 118, 347–353.
- Selkoe, D.J. (2002). Alzheimer's disease is a synaptic failure. *Science* 298, 789–791.
- Selkoe, D.J. American College of Physicians; American Physiological Society (2004). Alzheimer disease: mechanistic understanding predicts novel therapies. *Ann. Intern. Med.* 140, 627–638.
- Shi, Y., Inoue, H., Wu, J.C., and Yamanaka, S. (2016). Induced pluripotent stem cell technology: a decade of progress. *Nat. Rev. Drug Discov* 16, 115–130.
- Szabo, E., Rampalli, S., Risueño, R.M., Schnerch, A., Mitchell, R., Fiebig-Comyn, A., Levadoux-Martin, M., and Bhatia, M. (2010). Direct conversion of human fibroblasts to multilineage blood progenitors. *Nature* 468, 521–526.
- Thatava, T., Kudva, Y.C., Edukulla, R., Squillace, K., De Lamo, J.G., Khan, Y.K., Sakuma, T., Ohmine, S., Terzic, A., and Ikeda, Y. (2013). Inpatient variations in type 1 diabetes-specific iPS cell differentiation into insulin-producing cells. *Mol. Ther.* 21, 228–239.
- Vassar, R., Kuhn, P.H., Haass, C., Kennedy, M.E., Rajendran, L., Wong, P.C., and Lichtenthaler, S.F. (2014). Function, therapeutic potential and cell biology of BACE proteases: current status and future prospects. *J. Neurochem.* 130, 4–28.
- Vierbuchen, T., Ostermeier, A., Pang, Z.P., Kokubu, Y., Südhof, T.C., and Wernig, M. (2010). Direct conversion of fibroblasts to functional neurons by defined factors. *Nature* 463, 1035–1041.
- Vogt, M., Stumpfe, D., Geppert, H., and Bajorath, J. (2010). Scaffold hopping using two-dimensional fingerprints: true potential, black magic, or a hopeless endeavor? Guidelines for virtual screening. *J. Med. Chem.* 53, 5707–5715.
- Wallwey, C., and Li, S.-M. (2011). Ergot alkaloids: structure diversity, biosynthetic gene clusters and functional proof of biosynthetic genes. *Nat. Prod. Rep.* 28, 496–510.
- Walsh, D.M., Klyubin, I., Fadeeva, J.V., Cullen, W.K., Anwyl, R., Wolfe, M.S., Rowan, M.J., and Selkoe, D.J. (2002). Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* 416, 535–539.
- Willett, P., Barnard, J.M., and Downs, G.M. (1998). Chemical similarity searching. *J. Chem. Inf. Comput. Sci.* 38, 983–996.
- Yahata, N., Asai, M., Kitaoka, S., Takahashi, K., Asaka, I., Hioki, H., Kaneko, T., Maruyama, K., Saido, T.C., Nakahata, T., et al. (2011). Anti-A $\beta$  drug screening platform using human iPS cell-derived neurons for the treatment of Alzheimer's disease. *PLoS ONE* 6, e25788.
- Yan, R. (2016). Stepping closer to treating Alzheimer's disease patients with BACE1 inhibitor drugs. *Transl. Neurodegener.* 5, 13.
- Zhang, Y., Pak, C., Han, Y., Ahlenius, H., Zhang, Z., Chanda, S., Marro, S., Patzke, C., Acuna, C., Covy, J., et al. (2013). Rapid single-step induction of functional neurons from human pluripotent stem cells. *Neuron* 78, 785–798.